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(71) Applicant: SYNTELLO, INC. [US/US]; Suite 310, 4350 Executive Drive, San Diego, CA 92121 (US).

(72) Inventors: CZERKINSKY, Cecil; Linnegatan 5, S-413 04 Goteborg (SE). HOLMGREN, Jan; Korvettgatan 1D, S-421 74 Vastra Frolunda (SE). HORAL, Peter; Orangerigatan 21B, S-412 66 Goteberg (SE). SVENNERHOLM, Bo; Jakobsdalsgatan 48, S-412 66 Goteborg (SE). VAHLNE, Anders; Grimmerads By 10, S-421 70 Vastra Frolunda (SE).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson and Bear, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

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(54) Title: INHIBITION OF HIV MUCOSAL INFECTION

(57) Abstract

Disclosed is a method of inhibiting the entry of HIV into the vaginal and rectal mucosal epithelium by administering a peptide vaccine. Antibodies generated against peptides corresponding to epitopes of gp120 involved in entry into mucosal cells are generated *in vivo* by introduction of peptides or peptide-cholera toxin conjugates into epithelial cells. The resulting neutralizing antibodies are able to block subsequent infection of these tissues by HIV.

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INHIBITION OF HIV MUCOSAL INFECTION

FIELD OF THE INVENTION

This invention relates to the inhibition of binding of HIV to the genital and rectal mucosal epithelium.

BACKGROUND OF THE INVENTION

Dispersed aggregates of non-encapsulated lymphoid tissue are often localized to the submucosal areas of the gastrointestinal, respiratory and urogenital tracts. These tracts are a main means of entry into the body by foreign microorganisms. Secretory immunoglobulin A (IgA) is an antibody capable of crossing mucosal membranes and protecting them against invasion by pathogens. Mucosal lymphoid tissue thus plays an important role in the local immune response which occurs at mucosal surfaces.

It is well established that mucosal epithelial cells, regardless of whether they express the cell surface CD4 receptor used by the HIV to enter T-cells, macrophages and Langerhans cells, can be latently infected by HIV (Fantini et al., (1992) *J. Virol.*, 66: 5805; Fantini et al., (1991) *Virology*, 185: 904). Although the receptor(s) for HIV entry into mucosal intestinal epithelial cells appear to be glycolipids (Yahi et al., (1992) *J. Virol.*, 66: 4848), there is no information regarding the HIV epitope(s) which mediates attachment to these cells. Such knowledge would be of paramount importance since this epitope(s) would be an ideal target against which the local mucosal immune system could act to prevent the mucosal entry of HIV.

The induction of a mucosal immune response to prevent entry of human immunodeficiency virus (HIV-1) through the rectal and genital (vaginal) mucosa has not been significantly explored as an approach in preventing AIDS. Conventionally administered vaccines derived from the viral glycoprotein gp120 provide little immunity to HIV. Systemic immunization strategies have protected against intravenous challenge with simian immunodeficiency virus (SIV), the monkey counterpart of HIV, in monkeys, but have failed to prevent infection by SIV introduced via the vaginal mucosa (Miller et al., (1990) *J.*

Immunol., 144: 122).

In general, mucosal delivery of antigens does not evoke a strong immune response. A notable exception, however, is cholera toxin (CT), produced by the bacterium *Vibrio cholera*, which is among the strongest mucosal immunogens known. CT binds strongly to a glycosphingolipid called ganglioside GM1 on mucosal cell surfaces using its B subunit. Mucosal administration of minute amounts of antigens covalently linked to the B subunit (CTB) has been shown to elicit vigorous mucosal as well as extramucosal immune responses in experimental animals including nonhuman primates (Czerkinsky et al., (1989) *Infect. Immun.*, 57: 1072-1077; Liang et al., (1988) *J. Immunol.*, 141, 3781-3787; Lehner et al., (1992) *Science*, 258: 1365-1369; Holmgren et al., (1993) *Vaccine*, 11: 1179-1184). The possibility of disseminating a specific B-cell response from the gut to other mucosal tissues in orally immunized humans has also been documented (Czerkinsky et al., (1991) *Infect. Immun.*, 59: 996-1001). In addition, it has been demonstrated that mucosal immune responsiveness in HIV-1 infected individuals remains relatively stable compared to a dramatic hyporesponsiveness to parenterally administered vaccines (Eriksson et al., (1993) *AIDS*, 7: 1087-1091). This study not only underscores the relative independence of mucosal and systemic immunity, but also raises the possibility of inducing HIV-specific mucosal immunity in an already infected individual, thus interfering with subsequent mucosal transmission. Immunization strategies effective in inducing an immune response in the genital and rectal mucosa have been evaluated in nonhuman primates (Lehner et al., (1992) *Science*, 258: 1365-1369).

In view of the incidence of sexually transmitted HIV infection (over 75% of all cases), the alarming increase in the number of new AIDS cases and the inability of systemic immunization strategies to induce a significant mucosal immune response, a vaccine able to produce an immune response at the mucosal surfaces through which HIV gains access to the circulation would have significant value as part of an overall

approach to reducing HIV-1 infection.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a method for inhibiting the infection of mucosal cells by HIV-1 by administering a vaccine to the mucosa, thereby delivering to the mucosa a peptide of HIV-1 gp120 having from about 10 to about 50 amino acids, whereby antibodies against the peptide are generated in the mucosa, the peptide being selected such that the antibodies inhibit infection of HIV-1 in mucosal epithelial cells.

In another aspect of this preferred embodiment, the peptide includes an epitope effective to generate mucosal production of antibodies that inhibit infection of mucosal cells by HIV-1, the peptide consisting essentially of SEQ ID NOS: 9, 10, 11, 12, or 13. Advantageously, the vaccine further includes an agent for enhancing delivery of the peptide to the mucosa. Preferably, the agent is a mucosal binding protein; most preferably, it is either the binding subunit of cholera toxin or that of *E. coli* heat labile enterotoxin. The invention also provides that the peptide and the mucosal binding protein are bound together to form a chimeric protein which may advantageously be the expression product of recombinant DNA. In another embodiment of the invention, the agent is a lipid. Preferably, the lipid is in the form of a lipid vesicle. Another aspect of this preferred embodiment provides that the administering step comprises administering to the mucosa a polynucleotide operably encoding the peptide, whereby the peptide is produced by cells of the mucosa.

A further embodiment of the invention provides a vaccine for inhibiting the infection of mucosal cells by HIV-1, comprising a 10 to 50 amino acid peptide of HIV-1 gp120 having an epitope selected such that antibodies against this epitope inhibit the infection of mucosal epithelial cells by HIV-1, and a compound or structure associated with the peptide for facilitating delivery of the peptide to the mucosa. Preferably, this peptide consists essentially of SEQ ID NO 9,

10, 11, 12 or 13 and the compound or structure is a lipid vesicle. Most preferably, the compound or structure is a mucosal binding protein. In a particularly preferred embodiment, the binding protein is a cholera toxin protein which may advantageously be the binding subunit. In another aspect of this preferred embodiment, the binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses the identification of synthetic peptides derived from the sequence of the envelope glycoprotein gp120 of HIV-1. These peptides were used to generate neutralizing antibodies which inhibited infection of transformed human vaginal and colorectal cell lines *in vitro*. These peptides will induce the production of a localized mucosal immune response, generating antibodies able to neutralize infection of human colorectal and vaginal epithelial cells by HIV-1. The peptides are set forth herein as SEQ ID NOS: 9-13. In one aspect of the invention, one or more of the peptides of SEQ ID NO:9, 10, 11, 12, and 13 is used to generate antibodies. These antibodies can be generated in any conventional manner, including by intramuscular, intraperitoneal, subcutaneous, or mucosal administration to an animal. Generation of both monoclonal and polyclonal antibodies are contemplated. These antibodies are then used to prevent infection of cells of the mucosal epithelium by providing the antibodies in association with the mucosal cells and then challenging the cells with HIV-1. The antibodies inhibit or prevent binding of the virus to the cells, and thereby inhibit or prevent infection of the cells by the virus.

The antibodies can be exogenous or endogenous antibodies, and the cells can be *in vitro* or *in vivo*. When the cells are *in vitro*, the antibodies are typically generated in laboratory or domestic animals or are monoclonal antibodies. More importantly, it provides a valuable tool for analyzing the mechanism and structure involved in that binding.

When the cells are *in vivo*, the antibodies are preferably

endogenous mucosal antibodies that have been generated by administering one or more of the peptides of SEQ ID NOs 9-13 to the animal in which the cells are located. Mucosal vaccination, as described below, is particularly preferred. However, exogenous antibodies may also be administered to the animal to inhibit HIV-1 infection of mucosal cells. In all of the treatments described herein, the mucosal cells are preferably of human origin.

The peptides of the present invention can be utilized alone or in combination and can also be uncoupled or coupled to other epithelial cell binding proteins including CT, CTB and the binding subunit of *E. coli* heat labile enterotoxin. The peptides may be coupled by either chemical or recombinant means. DNA encoding the peptides can be joined to DNA encoding cholera toxin, or its B subunit, by well known methods, inserted into a eukaryotic expression vector and delivered to epithelial cells using lipid vesicles or lamellar structures. The production of these peptide-CT, CTB or enterotoxin conjugates *in vivo* will then elicit a localized mucosal immune response and will protect against subsequent infection by HIV. The inclusion of mucosal epithelial cell binding proteins, such as cholera toxin, will advantageously increase the efficiency of entry of peptides into these cells. Since the B subunit of the cholera toxin A-B dimer is responsible for binding to cell surface receptors, a peptide-CTB conjugate will also bind efficiently to epithelial cells. The literature also reports methods for forming compositions of immunogenic peptides and other gut binding proteins (Wenneras et al., (1990) *FEMS Microbiol. Lett.*, **66**: 107-112). Techniques for forming peptide-CTB conjugates are well known (Liang et al., (1988) *J. Immunol.*, **141**: 3781-3787; Sanchez et al., (1990) *Res. Microbiol.*, **141**: 971-979). Liposome formation and delivery of peptides encapsulated in liposomes is also well known as described by *Lowell (New Generation Vaccines*, Woodrow, G.C. and Levine, M.M., eds., Marcel Dekker, Inc., New York, pp. 141-160). These peptides are also useful in the production of monoclonal and polyclonal antibodies.

These antibodies have a distinct neutralizing effect on HIV-1. These peptides, either alone or after coupling to CT or other molecules, may be administered orally, rectally, vaginally, or in a combination of these routes in an amount sufficient to generate a mucosal antibody response sufficient to inhibit HIV-1 entry into the mucosal epithelial cells. The amounts of peptides used will depend on their pharmaceutical formulation and the site and route of delivery; however, for an adult human, a suitable immunogenic amount of peptide is generally between about 50 μg and about 1 mg, administered one to four times over a period of two weeks to one year or longer.

The peptides, peptide-binding protein conjugates, and other compositions of the present invention can be administered orally to generate a localized gastrointestinal mucosal immune response or intravaginally or intrarectally to produce a localized mucosal immune response in these areas prone to viral entry by sexual contact. These peptides can be administered in unit dosage in an amount necessary to produce localized mucosal immunity against HIV-1 infection. Pharmaceutical compositions envisioned for oral administration include tablets, capsules, liquids, and the like and those contemplated for intravaginal or intrarectal administration include injectable carriers, suppositories, ointments, gels, creams, foams, sprays, dispersions, suspensions, pastes and the like in an amount from about 10 μg to about 10 mg or more. These preparations can be in any suitable form, and generally comprise the active ingredient in combination with any of the well known pharmaceutically acceptable carriers. The preparations may further advantageously include preservatives, antibacterials, antifungals, antioxidants, osmotic agents, and similar materials in composition and quantity as is conventional. For assistance in formulating the compositions of the present invention, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton, PA (1975).

The contemplated modes of administration assume that the peptides or conjugates are able to be directly taken up by the

epithelial cells lining these areas. These peptides and conjugates may advantageously be enclosed in liposomes to facilitate delivery of these agents to cells. Direct injection of the peptides or peptide-binding protein conjugates, either alone or in combination with lipid vesicles or other lamellar structures, into the mucosal endothelium in a similar dose range is also envisioned as a method of eliciting an anti-HIV response in these tissues.

Example 1

Susceptibility of colorectal and vaginal epithelial cells to infection by HIV-1

HIV-1 infectious virus stocks of HTLV-IIIB-infected H9 T cell lymphoma cells (ATCC HTB-176) (Popovic et al., (1984) *Science*, **224**: 497-500) were used in the following experiments. The cells were maintained in RPMI-1640 medium containing 20% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin. Virus stocks were prepared using well known procedures and frozen at -90°C. One stock of HTLV-IIIB with endpoint titer of 1 x 10^4 tissue culture infectious doses ($TCID_{50}$) was used for all experiments.

Endpoint titration of the HTLV-IIIB isolate of HIV-1 in two clones of transformed vaginal epithelial cells (Hs 760 T and Hs 769.Vg cells; ATCC CRL-7491 and 7499, respectively) and 12 subclones of colon adenocarcinoma HT-29 cells (ATCC HTB-38) were performed by inoculation of respective cell lines with serial 10-fold dilutions of virus with 100 μl/well (ranging from 1 $TCID_{50}$/cell to 0.00001 $TCID_{50}$/cell) in 24-well plates (Costar) for 2 hours at 37°C. After adsorption, cells were washed five times with Modified Eagle's Medium (MEM) and supplemented with 1.5 ml growth medium (DMEM for vaginal cells and MEM for colon cells, both containing 10% fetal calf serum (FCS), 1% L-glutamine, and antibiotics). Seven days after infection, epithelial cells were washed five times with MEM and treated with 0.1% trypsin in phosphate buffered saline (PBS) for 5 min at 37°C. HTLV-IIIB-infected H9 cells (10^6 cells) in H9 maintenance medium were added to each well and cocultured with epithelial cells for 24 hours. The H9

cultures were microscopically followed for 7 days for the presence of HIV-induced syncytium formation and p24 antigen production using an ELISA able to detect as little as 100 pg p24/ml). The vaginal cell lines Hs 760.T and Hs 769.Vg were permissive. Viral infection of Hs 760.T was detected by coculture at a high multiplicity (1 $TCID_{50}$/cell) and 6 of the HT-29 colon cell clones were permissive at multiplicities ranging from 0.1-0.01 $TCID_{50}$/cell. Of these clones, clo0ne L20 was chosen for further study.

Table 1

Susceptibility of colorectal and vaginal epithelial cells to infection by HIV-1 (HTLV-IIIB).

			multiplicity of infection (TCID50/cell)			
cell Line	subclone	method*	1	10^{-1}	10^{-2}	$x10^{3}$
HT-29	E0	coculture	-	-	-	-
"	E5	coculture	-	-	-	-
"	E8	coculture	-	-	-	-
"	L2	coculture	-	-	-	-
"	L4	coculture	-	-	-	-
"	L16	coculture	-	-	-	-
"	L18B	coculture	+	-	-	-
"	L18A	coculture	+	+	-	-
"	L12	coculture	+	+	-	-
"	L10	coculture	+	+	+	-
"	L14	coculture	+	+	+	-
"	L20	coculture	+	+	+	-
"	L20	PCR	2.5**	2	0.5	0.125
HS 769.Vg		coculture	-	-	-	-
"		PCR	2.5	0.6	0.5	0.125
HS 760.T		coculture	+	-	-	-
"		PCR	10	-	-	-

* coculture with H9 cells and subsequent p24 antigen detection or detection of proviral DNA by PCR.

** copy number, $x10^{-2}$ per cell

HIV-1 RNA and DNA was detected both in epithelial cells and in the culture supernatants as described in the following examples.

Example 2

Detection of proviral DNA by PCR

Epithelial cells were harvested seven days post-infection and DNA was extracted (Sambrook et al., (1989) *Molecular Cloning*, second edition, Cold Spring Harbor Laboratory Press, 2: 9.16-9.19). Primers specific for the HIV-1 *env* gene (5'-GTAACGCACAGTTTTAATTGTGGAGGGGAA-3'; SEQ ID NO: 1) and (5'-CCTCATATTTCCTCCTCCAGGTCT-3'; SEQ ID NO: 2) were used for detection of proviral DNA. DNA (200 ng) was amplified on a DNA thermal cycler (Perkin-Elmer, Norwalk, CT) using α-^{32}P-dCTP to label the fragments. The reaction mixture consisted of 10 μl of 10x PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 20 pmol primers, 0.125 mM dNTPs, 5 μCi α-^{32}P-dCTP and 0.5 units *Taq* DNA polymerase (Promega). The amplification was for 35 cycles and included denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. One-tenth of the final reaction mixture was analyzed by electrophoresis on 5% polyacrylamide gels. The gels were dried and exposed to X-ray film (X-OMAT; Eastman Kodak, Rochester, NY) for 13-16 hours using an intensifying screen.

To measure HIV copy number (the number of HIV genomes), two-fold serial dilutions of DNA isolated from ACH-2 cells, which contain one proviral copy per cell (Clouse et al., (1989) *J. Immunol.*, **142**: 431-438; Seshamma et al., (1992) *J. Virol. Methods*, **40**: 331-346; Graziosi et al., (1993) *Proc. Natl. Acad. Sci. U.S.A.*, 90: 6405-6409). The total amount of DNA in each dilution was normalized to 200 ng using DNA extracted from H9 cells and PCR was performed as above. HIV copy number was estimated by comparison of the intensities of the amplified bands. PCR analysis using a pair of human β-actin primers was performed in parallel as an internal standard.

Example 3

Detection of HIV RNA expression by RT-PCR

Epithelial cells were harvested 7 days post-infection and total RNA was extracted by the RNAzol method (Biotex Laboratories, Houston, TX). For each sample, 500 ng of total RNA was incubated with 10 units RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany) at 37°C for 1 hour. Samples were then heated to 80°C for 10 min to degrade the DNase. cDNA was synthesized in a reverse transcriptase (RT) reaction with 10 pmol downstream PCR primer (described below), 0.625 mM dNTPs, 5 x reaction buffer (Promega) and 200 units Moloney murine leukemia virus RT (Promega) to a final volume of 20 μl. The mixture was incubated at 37°C for 1 hour. The cDNA was amplified for 30 cycles by PCR as described in Example 3. The primers used to detect HIV-1 regulatory RNA were as follows:

5'-GAAGAAGCGGAGACAGCGACG-3' (SEQ ID NO: 3)

5'-GGCCTGTCGGGTCCCCTCG-3' (SEQ ID NO: 4)

Primers specific for the HIV-1 major splice donor (MSD) site used to detect HIV-1 structural RNA were as follows:

5'-CTCTCGACGCAGGACTCGGC-3' (SEQ ID NO: 5)

5'-CTTTCCCCCTGGCCTTAACCG-3' (SEQ ID NO: 6)

$_{32}$P-dCTP was incorporated into the amplified fragments and one-tenth of the final reaction mixture was analyzed by electrophoresis on 8% polyacrylamide gels. In each sample, RNA without reverse transcriptase was also amplified by PCR to demonstrate that the amplified fragments were from HIV cDNA, not from contamination of HIV DNA.

Example 4

Detection of HIV RNA in culture supernatants by RT-nested PCR

RNA was extracted from 500 μl of culture supernatants by the RNAzol method. After DNase I treatment, cDNA was synthesized by a RT reaction with SEQ ID NO:8 as an RT primer. The synthesized cDNA was amplified by the nested PCR method. The primers for the first PCR were as follows:

5'-GAAGAAGAGATAGTAATTAGATCT-3' (SEQ ID NO: 7)

5'-GGTGGGTGCTACTCCTAATTGTTCAATTC-3' (SEQ ID NO: 8)

The primers used for the second (nested) PCR were SEQ ID

NO: 7 and SEQ ID NO: 2 . One-tenth of the first PCR product was added to the second PCR reaction. The PCR conditions were as described in Example 3, except that 40 cycles of amplification were performed. One-tenth of the final reaction mixture was analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide. RNA samples without RT were also amplified by the nested primers as a test for DNA contamination. DNA content and RNA expression in HIV-1 infected epithelial cells is shown in Table 2.

Approximately 1% of HT29 L20 cells are infected with HIV-1, if HIV-infected cells contain 1 copy of proviral DNA per cell. As can be seen in Table 2, expression of regulatory RNA in HIV-1 infected HT29 L20 cells is lower than that in HIV-1 infected H9 cells and ACH-2 cells. Expression of structural RNA is barely detectable.

Table 2

HIV-1 (HTLV-IIIB) RNA expression in colorectal and vaginal epithelial cells

cell line	HIV-1 DNA (copy/cell)	HIV-1 RNA expression		Virus in cell medium	
		Regulatory RNA	Structural RNA	HIV RNA	P24 antigen
HT-29 clone L20	0.025	+	+	-	-
Hs 769.Vg	0.025	+	+	-	-
Hs 760.T	0.1	+	+	-	-
ACH-2	1	+	+	+	+
H-9	20	+	+	+	+

Example 6

Neutralization of HTLV-IIIB infectivity in L20 and Hs769 cells by antipeptide antisera

Hyperimmune sera was isolated from monkeys immunized with the five peptides derived from the gp120 sequence listed below (Table 3).

Table 3

peptide no.	sequence	SEQ ID NO.
12	GEIKNCSFNISTSIRGKVQKEYAFF	9
15	LTSCNTSVITQACPKVSFEPIPIHYC	10
16	PKVSFEPIPIHYCAPAGFAILKCNN	1
19	THGIRPVVSTQLLLNGSLAEEE	12
24	IRIQRGPGRAFVTIGKIGNMRQAH	13

Solid phase peptide synthesis was performed using an Applied Biosystems (Foster City, CA) 430A peptide synthesizer. An amino-terminal cysteine residue was added to each peptide to facilitate coupling to a carrier protein. Peptides were covalently coupled to ovalbumin, grade V (Sigma, St. Louis, MO) at an approximate 10:1 (peptide:ovalbumin) molar ratio using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pharmacia, Uppsala, Sweden). Three to five year old male and female monkeys (*Macaca fascicularis)* were immunized by three consecutive intramuscular injections of 100 μg ovalbumin-conjugated peptides emulsified in Freund's complete (first injection) or incomplete (second and third injections) given three weeks apart. Blood was collected from the femoral vein before immunization and one or two weeks after the final immunization. Pre- and post-immune sera were prepared and stored at -20°C.

These peptides have been shown to elicit neutralizing antibodies to HIV in monkeys (Vahlne et al., (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 10744-10748). These antibodies, a guinea pig hyperimmune serum with high neutralizing HTLV-IIIB capacity and a monoclonal antibody against gp120 (the latter two kindly provided by L. Akerblom, Uppsala, Sweden) were assayed for their ability to neutralize HTLV-IIIB infectivity by primary inhibition of HIV-1 infectivity in HT-29, clone L20, colon cells and in Hs 760.T vaginal cells and subsequently assayed by cocultivation with highly permissive H9 lymphoid cells.

Stock virus was diluted to 10^4 $TCID_{50}$ for neutralization in colon cells and used undiluted (10^6 $TCID_{50}$) for

neutralization in vaginal cells and mixed with serial four fold dilutions of heat-inactivated monkey sera starting at 1:5. The monkey sera were used at a final dilution of 1:10 or 1:20. The guinea pig hyperimmune serum served as a positive control. After incubation for 2 hours at 37°C, the serum virus mixture was incubated with the epithelial cells for 2 hours at 37°C. The cells were washed twice with medium and supplemented with 1.5 ml of respective maintenance medium/well. Seven days after infection the cells were washed five times and treated with 0.1% trypsin at 37°C for 5 minutes. H9 cells (10^6) were added to each well and cocultures were monitored for 7 days for syncytia formation and presence of p24 antigen. Results for HS 760.T cells, Hs769 cells and HT-29 L20 cells are indicated in Tables 4/5, 6, and 7, respectively, and are expressed as mean neutralization titers, defined as the reciprocal of the serum dilution that reduced the p24 antigen by at least 90%. The HIV-1 copy number is also shown for HIV-1 infected HS 760.T cells (Tables 4 and 5).

Table 4

Neutralization of HIV-1 (HTLV-IIIB) infectivity in Hs 760.T cells by monkey hyperimmune sera against gp120 peptides.

	HIV-1 DNA (copy/10^4cells)		neutralization assayed by cocultivation	
sera to peptides	pre-immune	post-immune	pre-immune	post-immune
gp120-12	125	<12.5	ND	+
gp120-15	ND	<12.5	ND	+
gp120-16	100	25	ND	+
gp120-19	125	<12.5	ND	+
gp120-24	100	<12.5	ND	+
mixture gp120-(12+15+16+19+24)	125	<12.5	-	+
Guinea pig anti-gp120	125	<12.5	-	+

ND, not done

Table 5

Neutralization of HIV-1 (HTLV-IIIB) infectivity in Hs 760.T cells by guinea pig anti-gp120 serum and monkey hyperimmune sera against gp120 peptides.

	HIV-1 DNA (copy/3x10⁴cells)		neutralization assayed by cocultivation	
Serum*	Pre-immune	Post-immune	Pre-immune	Post-immune
Guinea pig anti-gp120	375	<37.5	-	+
gp120-12	375	<37.5	ND	+
gp120-15	ND	<37.5	ND	+
gp120-16	300	75	ND	+
gp120-19	375	<37.5	ND	+
gp120-24	300	<37.5	ND	+
mixture gp120-(12+15+16+19+24)	375	<37.5	-	+

* Guinea pig anti-gp120 serum and monkey hyperimmune sera against gp120 peptides were tested at a final dilution of 1/40 and 1/10, respectively. Mixture of gp120 peptides antisera was tested at a final dilution of 1/20. ND, not done.

Table 6

Neutralization of HIV-1 (HTLV-IIIB) infectivity in HS 769.Vg cells by guinea pig and monkey hyperimmune sera against gp120.

Guinea pig anti gp120

dilution	HIV-1 copy number (copy/10⁴ cells)	
	pre-immune	post-immune
x40	500	<12.5
x160	250	100
x640	250	100

Monkey anti-peptide 24

dilution	HIV-1 copy number (copy/10⁴ cells)	
	pre-immune	post-immune
x10	500	12.5
x40	250	25

Table 7

Neutralization of HIV-1 (HTLV-IIIB) infectivity in HT-29 L20 cells by guinea pig anti-gp 120 serum and monkey hyperimmune sera against gp120 peptides.

	Neutralization assayed		
	by cocultivation		by PCR
Serum	Pre-immune	Post-immune	Post-immune
Guinea pig anti-gp120	-	+	+
gp120-1 to gp120-11 (aa 1-164)	-	-	ND
gp120-12 (aa 152-176)	-	+	+
gp120-13 to gp120-14 (aa 165-205)	-	-	ND
gp120-15 (aa 193-218)	-	+	+
gp120-16 (aa 206-230)	-	+	+
gp120-17 to gp120-18 (aa 219-257)	-	-	ND
gp120-19 (aa 248-269)	ND	ND	ND
gp120-20 to gp120-23 (aa 258-320)	-	-	ND
gp120-24 (aa 307-330)	-	+	+
gp120-25 to gp120-40 (aa 321-511)	-	-	ND
mixture of gp120-(12+15+16+19+24)	-	+	ND

ND, not done.

The results indicated that the level of proviral DNA was markedly decreased by incubation of HT29 L20 cells with anti-gp120 guinea pig serum. A decrease in viral load was also detected in cells incubated with the antisera to peptides corresponding to SEQ ID NOS: 9, 10, 11 and 13 (Table 3). HIV-1 copy number was also markedly decreased in HS769 vaginal epithelial cells by an antiserum to the peptide of SEQ ID NO: 13.

Example 7

Protection from HIV-1 mucosal infection *in vivo* with a vaccine against gp120 epitopes

DNA corresponding to peptides having the sequence of SEQ ID NO: 9-13 is linked to DNA encoding the B subunit of cholera toxin by standard methods of molecular biology. The resulting chimeric construct is placed in a commercially available eukaryotic expression vector such as pGEX (Pharmacia, Piscataway, NJ) containing the appropriate translation initiation and termination signals. This construct is then incorporated into a lipid vesicle by methods well known in the art. The lipid vesicle is then formulated into a foam or suppository composition by well known pharmacolological preparation methods and administered vaginally and/or rectally to humans at high risk for HIV infection. The dose range administered is in the range of from about 10 μg to 10 mg. The administration is repeated at two week intervals for a total of three administrations. The presence of anti-HIV antibodies in the vaginal and rectal mucosa is assayed by isolating protein from vaginal secretions and feces (which contains cells shed from the vaginal and rectal epithelium, respectively) and performing a p24 ELISA to determine whether any antibodies are present. These antibodies can then be used in HIV-1 virus neutralization assays (Vahlne et al., (1991) *Proc. Natl. Acad. Sci. U.S.A.*, 88: 10744-10748).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SYNTELLO, Inc.

(ii) TITLE OF INVENTION: Inhibition of HIV Mucosal Infection

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Knobbe, Martens, Olson & Bear
(B) STREET: 620 Newport Center Drive, Sixteenth Floor
(C) CITY: Newport Beach
(D) STATE: CA
(E) COUNTRY: U.S.A.
(F) ZIP: 92660

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Israelsen, Ned A.
(B) REGISTRATION NUMBER: 29.655
(C) REFERENCE/DOCKET NUMBER: METRICS.036QPC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 235-8550
(B) TELEFAX: (619) 235-0176

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTAACGCACA GTTTTAATTG TGGAGGGGAA 30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTCATATTT CCTCCTCCAG GTCT 24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGAAGCGG AGACAGCGAC G 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCCTGTCGG GTCCCCTCG 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCTCGACGC AGGACTCGGC 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTTCCCCCT GGCCTTAACC G 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAAGAAGAGA TAGTAATTAG ATCT 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTGGGTGCT ACTCCTAATT GTTCAATTC 29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Glu Ile Lys Asn Cys Ser Phe Asn Ile Ser Thr Ser Ile Arg Gly
1 5 10 15

Lys Val Gln Lys Glu Tyr Ala Phe Phe
20 25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Thr Ser Cys Asn Thr Ser Val Ile Thr Gln Ala Cys Pro Lys Val
1 5 10 15

Ser Phe Glu Pro Ile Pro Ile His Tyr Cys
20 25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Pro Lys Val Ser Phe Glu Pro Ile Pro Ile His Tyr Cys Ala Pro Ala
1 5 10 15

Gly Phe Ala Ile Leu Lys Cys Asn Asn
20 25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Thr His Gly Ile Arg Pro Val Val Ser Thr Gln Leu Leu Leu Asn Gly
1 5 10 15

Ser Leu Ala Glu Glu Glu
20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ile Arg Ile Gln Arg Gly Arg Gly Arg Ala Phe Val Thr Ile Gly Lys
1 5 10 15

Ile Gly Asn Met Arg Gln Ala His
20

WHAT IS CLAIMED IS:

1. A method for inhibiting the infection of mucosal cells by HIV-1, comprising the step of administering a vaccine to the mucosa, thereby delivering to the mucosa a peptide of HIV-1 gp120 having from about 10 to about 50 amino acids, whereby antibodies against said peptide are generated in said mucosa, said peptide being selected such that said antibodies inhibit infection of mucosal epithelial cells by HIV-1.

2. The method of Claim 1, wherein said peptide includes an epitope effective to generate mucosal production of antibodies that inhibit infection of said mucosal cells by HIV-1, said peptide consisting essentially of SEQ ID NOS: 9, 10, 11, 12, or 13.

3. The method of Claim 2, wherein said vaccine further comprises an agent for enhancing delivery of said peptide to the mucosa.

4. The method of Claim 3, wherein said agent comprises a mucosal binding protein.

5. The method of Claim 4, wherein said mucosal binding protein is the binding subunit of cholera toxin.

6. The method of Claim 4, wherein said mucosal binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

7. The method of Claim 4, wherein said peptide and said mucosal binding protein are bound together to form a chimeric protein.

8. The method of Claim 7, wherein said chimeric protein is the expression product of recombinant DNA.

9. The method of Claim 3, wherein said agent comprises a lipid.

10. The method of Claim 9, wherein said lipid is in the form of a lipid vesicle.

11. The method of Claim 1, wherein said administering step comprises administering to the mucosa a polynucleotide operably encoding said peptide, whereby said peptide is produced by cells of the mucosa.

12. A vaccine for inhibiting the infection of mucosal cells by HIV-1, comprising:

a 10 to 50 amino acid peptide of HIV-1 gp120 having an epitope selected such that antibodies against such epitope inhibit the infection of mucosal epithelial cells by HIV-1; and

a compound or structure associated with said peptide for facilitating delivery of said peptide to the mucosa.

13. The vaccine of Claim 12, wherein said peptide consists essentially of SEQ ID NO: 9, 10, 11, 12 or 13.

14. The vaccine of Claim 12, wherein said compound or structure is a lipid vesicle.

15. The vaccine of Claim 12, wherein said compound or structure is a mucosal binding protein.

16. The vaccine of Claim 15, wherein said binding protein is a cholera toxin protein.

17. The vaccine of Claim 15, wherein said binding protein is the binding subunit of cholera toxin.

18. The vaccine of Claim 15, wherein said binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

19. A peptide of HIV-1 gp120 having from about 10 to about 50 amino acids for use in inhibiting the infection of mucosal epithelial cells by HIV-1, wherein said peptide is administered to the mucosa, said peptide being selected such that antibodies against said peptide are generated in said mucosa, said antibodies inhibiting infection of said mucosal epithelial cells by said HIV-1.

20. The peptide of Claim 19, wherein said peptide includes an epitope effective to generate mucosal production of antibodies that inhibit infection of said mucosal cells by HIV-1, said peptide consisting essentially of SEQ ID NOS: 9, 10, 11, 12, or 13.

21. The peptide of Claim 20, wherein said peptide further comprises an agent for enhancing delivery of said peptide to the mucosa.

22. The peptide of Claim 21, wherein said agent comprises a mucosal binding protein.

23. The peptide of Claim 22, wherein said mucosal binding protein is the binding subunit of cholera toxin.

24. The peptide of Claim 22, wherein said mucosal binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

25. The peptide of Claim 22, wherein said peptide and said mucosal binding protein are bound together to form a chimeric protein.

26. The peptide of Claim 25, wherein said chimeric protein is the expression product of recombinant DNA.

27. The peptide of Claim 21, wherein said agent comprises a lipid.

28. The peptide of Claim 27, wherein said lipid is in the form of a lipid vesicle.

29. The peptide of Claim 19, wherein said administering step comprises administering to the mucosa a polynucleotide operably encoding said peptide, whereby said peptide is produced by cells of the mucosa.

AMENDED CLAIMS

[received by the International Bureau on 30 March 1995 (30.03.95); original claims 1,2,12,13 and 20 amended; new claims 30-33 added; remaining claims unchanged (3 pages)]

1. A method for inhibiting infection of mucosal epithelium cells by HIV-1, comprising the step of administering a vaccine to the epithelium, said vaccine comprising a peptide of HIV-1 gp120 having from about 10 to about 50 amino acids, whereby antibodies against said peptide are generated in said mucosa, said peptide being selected such that said antibodies inhibit infection of said cells by HIV-1.

2. The method of Claim 1, wherein said peptide includes an epitope effective to generate mucosal production of antibodies that inhibit binding between said mucosal cells and HIV-1, said epitope being found in SEQ ID NO: 9, 10, 11, 12 or 13.

3. The method of Claim 2, wherein said vaccine further comprises an agent for enhancing delivery of said peptide to the mucosa.

4. The method of Claim 3, wherein said agent comprises a mucosal binding protein.

5. The method of Claim 4, wherein said mucosal binding protein is the binding subunit of cholera toxin.

6. The method of Claim 4, wherein said mucosal binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

7. The method of Claim 4, wherein said peptide and said mucosal binding protein are bound together to form a chimeric protein.

8. The method of Claim 7, wherein said chimeric protein is the expression product of recombinant DNA.

9. The method of Claim 3, wherein said agent comprises a lipid.

10. The method of Claim 9, wherein said lipid is in the form of a lipid vesicle.

11. The method of Claim 1, wherein said administering step comprises administering to the mucosa a polynucleotide operably encoding said peptide, whereby said peptide is produced by cells of the mucosa.

12. A composition for inhibiting the infection of mucosal cells by HIV-1, comprising:

a 10 to 50 amino acid peptide of HIV-1 gp120 having an epitope selected such that antibodies against such epitope inhibit the binding of HIV-1 to mucosal epithelial cells; and

a compound or structure associated with said peptide for facilitating delivery of said peptide to the mucosa.

13. The composition of Claim 12, wherein said epitope is found in SEQ ID NO: 9, 10, 11, 12 or 13.

14. The composition of Claim 12, wherein said compound or structure is a lipid vesicle.

15. The composition of Claim 12, wherein said compound or structure is a mucosal binding protein.

16. The composition of Claim 15, wherein said binding protein is a cholera toxin protein.

17. The composition of Claim 15, wherein said binding protein is the binding subunit of cholera toxin.

18. The composition of Claim 15, wherein said binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

19. A peptide of HIV-1 gp120 having from about 10 to about 50 amino acids for use in inhibiting the infection of mucosal epithelial cells by HIV-1, wherein said peptide is administered to the mucosa, said peptide being selected such that antibodies against said peptide are generated in said mucosa, said antibodies inhibiting infection of said mucosal epithelial cells by said HIV-1.

20. The peptide of Claim 19, wherein said peptide includes an epitope effective to generate mucosal production of antibodies that inhibit infection of said mucosal cells by HIV-1, said epitope being found in SEQ ID NO: 9, 10, 11, 12 or 13.

21. The peptide of Claim 20, wherein said peptide further comprises an agent for enhancing delivery of said peptide to the mucosa.

22. The peptide of Claim 21, wherein said agent comprises a mucosal binding protein.

23. The peptide of Claim 22, wherein said mucosal binding protein is the binding subunit of cholera toxin.

24. The peptide of Claim 22, wherein said mucosal binding protein is the binding subunit of *E. coli* heat labile enterotoxin.

25. The peptide of Claim 22, wherein said peptide and said mucosal binding protein are bound together to form a chimeric protein.

26. The peptide of Claim 25, wherein said chimeric protein is the expression product of recombinant DNA.

27. The peptide of Claim 21, wherein said agent comprises a lipid.

28. The peptide of Claim 27, wherein said lipid is in the form of a lipid vesicle.

29. The peptide of Claim 19, wherein said administering step comprises administering to the mucosa a polynucleotide operably encoding said peptide, whereby said peptide is produced by cells of the mucosa.

30. A method for inhibiting infection of mucosal cells by HIV-1, comprising the steps of:

generating mucosal antibodies against one or more of the peptides of SEQ ID NO: 9, 10, 11, 12 or 13;

providing said antibodies in contact with mucosal epithelial cells; and

contacting said cells with HIV-1, whereby said antibodies inhibit the infection of said cells by HIV-1.

31. The method of Claim 30, wherein said antibodies are generated by administering an immunogen to mucosal tissue *in vivo*.

32. The method of Claim 31, wherein said immunogen is coupled to a mucosal binding protein.

33. The method of Claim 32, wherein said mucosal binding protein is the B subunit of cholera toxin.

STATEMENT UNDER ARTICLE 19

Vahlne et al. disclose coupling the peptides shown in SEQ ID NOS: 9-13 to carrier proteins. These conjugates inhibited syncytia formation and other parameters of HIV infection. Vahlne et al. disclose neither inhibition of mucosal infection nor coupling of the peptides shown in SEQ ID NOS: 9-13 to carrier proteins. Lehner et al. describe the generation of mucosal immunity by administration of the entire SIV p27 gag protein conjugated to CTB, along with a retrotransposon. Czerkinsky et al. demonstrate production of a mucosal immune response against CTB and *S. mutans* antigen covalently coupled to CTB. The Czerkinsky et al. references do not disclose or suggest generation of antibodies against HIV or HIV-derived peptides, either by direct administration or by coupling to CTB. Lehner et al. neither teach nor suggest that peptides can be used in their method. In addition, p27 gag and gp120 are completely different proteins and would not necessarily elicit similar antigenic responses.

It cannot be assumed that a second protein unrelated to a first protein, much less a peptide derived from that second protein, will generate a mucosal immune response. The instant claims recite the use of the peptide itself as a vaccine. In contrast, Lehner et al. disclose that p27 is advantageously administered in combination with CTB due to the good adjuvant properties of CTB. Czerkinsky et al. also stress the strong adjuvant properties of orally administered CTB. The use of CTB does not provide motivation to administer the peptide alone as a vaccine, since no adjuvant would be present and a good localized immune response would not be expected. Similarly, Czerkinsky et al. teach administration of a complete bacterial antigen coupled to CTB as well as the administration of CTB itself for the generation of mucosal immunity. Again, this does not provide motivation to administer a gp120-derived peptide alone as a vaccine.

For the reasons set forth above, Applicant submits that the claims patentably define over the prior art.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12152

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/12, 38/00; C07K 1/00
US CL : 424/184.1, 450; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 450; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, search terms: HIV, cholera toxin, peptide, mucosal immunity, liposomes

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 258, issued 20 November 1992, T. Lehner et al., "Induction of Mucosal and Systemic Immunity to a Recombinant Simian Immunodeficiency Viral Protein", pages 1365-1369, see entire article.	1-12, 19, 29
Y	Infection and Immunity, Volume 59, No. 3, issued March 1991, C. Czerkinsky et al., "Antibody-Producing Cells in Peripheral Blood and Salivary Glands After Oral Cholera Vaccination of Humans", pages 996-1001, see entire article.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 JANUARY 1995	02 FEB 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	LYNETTE F. SMITH
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12152

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 88, issued December 1991, A. Vahlne et al., "Immunizations of Monkeys With Synthetic Peptides Disclose Conserved Areas On gp120 of Human Immunodeficiency Virus Type I Associated With Cross-Neutralizing Antibodies and T-Cell Recognition", pages 10744-10748, see entire article.	13-18, 20-22
Y	Infection and Immunity, Volume 57, No. 4, issued April 1989, C. Czerkinsky et al., "Oral Administration Of A Streptococcal Antigen Coupled To Cholera Toxin B Subunit Evokes Strong Antibody Responses In Salivary Glands and Extramucosal Tissues", pages 1072-1077, see entire article.	22-25
Y	Immunology Today, Volume 11, issued 1990, G. Gregoriadis, "Immunological Adjuvants: A Role For Liposomes", pages 89-97, especially pages 93-94.	27-28
Y	US, A, 4,808,700 (ANDERSON ET AL.) 28 February 1989, abstract and columns 23-34.	5-11, 15- 18, 23-29